Activation of the transcription factor NF- κ B in the rat air pouch model of inflammation

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Abstract

Objective—To investigate the activation of NF- κ B in the carrageenan rat air pouch model of inflammation in a time course experiment, and the effect of dexamethasone on NF- κ B activation.

Methods—Air pouch tissue treated with carrageenan (inflamed tissue) was obtained from rats killed at days 1, 2, 3, 6, 14, 21, 28 and 35 after carrageenan challenge. Tissue was also taken from non-carrageenan treated pouches (non-inflamed tissue) at day 3, and from inflamed tissues treated with dexamethasone. Tissue sections were wax embedded and stained with an “activity specific” monoclonal antibody raised against the nuclear localisation signal (NLS) of the p65 sub-unit of NF- κ B.

Results—Cells containing activated NF- κ B were detected in the intimal and sub-intimal regions of the air pouches as early as day 1. There was a significant increase in cells staining for activated NF- κ B as the inflammation progressed. Initially cells staining were more prominent in the intimal versus sub-intimal region ($p < 0.001$ for day 1) and at later time points the pattern was reversed ($p < 0.001$ for day 6). There was a significant reduction in the number of cells staining for activated NF- κ B in tissue taken from dexamethasone treated rats, compared with inflamed pouches alone ($p < 0.001$). At no time point was positive staining for activated NF- κ B observed in blood vessels.

Conclusions—Activated NF- κ B is present in the inflamed air pouch and the activation is associated with the inflammatory response to carrageenan. Treatment with dexamethasone resulted in reduced numbers of cells staining for activated NF- κ B.

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The transcription factor nuclear factor κ B (NF- κ B) is a ubiquitous, heterodimeric DNA binding protein that induces the expression of a wide range of inflammatory genes. The target genes for NF- κ B include many genes involved in the inflammatory response, such as interleukin 1 (IL1), interleukin 6, tumour necrosis factor α (TNF α), and inducible nitric oxide synthase (for review see Baeuerle and Henkel¹).

The most common DNA binding form of NF- κ B is a heterodimer of p50 and p65 (Rel A) subunits. In non-stimulated cells, NF- κ B exists in the cytoplasm as a p50/p65 dimer bound to its specific inhibitory protein I κ B. Activating stimuli such as TNF α and IL1, trigger the dis-

sociation of I κ B from the NF- κ B/I κ B complex, and the subsequent degradation of I κ B. This dissociation exposes the NLS of p50 and p65, leading to the translocation of the subunits to the nucleus where they bind to DNA to initiate transcription of specific genes.¹

The air pouch model of inflammation was first described by Selye in 1953² and has been used to study both the mechanisms underlying the inflammatory response and various anti-inflammatory treatments. The non-inflamed cavity is lined by a thin layer of fibroblasts and macrophage-like cells and resembles the normal synovium.^{3,4} The introduction of an irritant into the cavity induces a chronic inflammatory response that resembles that seen in the rheumatoid joint.

We have previously demonstrated the activation of NF- κ B in the rheumatoid synovium.^{5,6} The tissue distribution of NF- κ B staining seemed to be related to acute and chronic phases of inflammation. We therefore used a time course experiment to study the distribution of NF- κ B staining as the inflammatory reaction progressed, and the effect of the corticosteroid, dexamethasone, on this process. Unlike some other animal models, the air pouch system allows the ready analysis of both tissue and exudate fluid over a time course. In this study, we show that this is a useful in vivo model for future investigations of NF- κ B dependent inflammatory mechanisms and the effects of anti-inflammatory drugs.

Methods

THE AIR POUCH

Fifty male Wistar rats were divided into three groups (1) non-inflamed (control) pouch (n=5), (2) inflamed (carrageenan treated) pouch (n=40) (further subdivided into eight groups (n=5) to form the time course) and (3) dexamethasone treated inflamed pouch (n=5). The air pouch was induced by injecting 20 ml of sterile air subcutaneously into the dorsum

Table 1 Mean number of cells staining positive for activated NF- κ B in the intimal and sub-intimal zones of the air pouch lining tissue, during the course of the inflammatory response. Cell counting was carried out as described in the Methods section

Day	Mean (SD) number of cells staining per μm^2 in the intimal zone	Mean (SD) number of cells staining per μm^2 in the sub-intimal zone	p Values for the difference between the two zones
1	12.4 (2.1)	2.2 (1.4)	$p < 0.001$
2	18.9 (8.9)	4.2 (1.1)	$p < 0.01$
3	7.0 (2.7)	6.3 (0.8)	$p = 0.58$
6	3.9 (1.0)	9.2 (1.1)	$p < 0.001$
14	0.2 (0.3)	6.7 (1.9)	$p < 0.01$
21	1.6 (0.4)	0.4 (1.0)	$p < 0.001$
28	2.6 (0.4)	—	—
35	1.0 (0.5)	—	—

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and maintained by re-injection two days later. The pouches were allowed to stabilise for 3 days, then (1) the carrageenan time course was begun with the injection of 1 ml of 2% carrageenan solution into the pouch (this was

defined as day 0, at day 1 one group of rats were killed, and this was repeated at days 2, 3, 6, 14, 21, 28 and 35), and (2) the dexamethasone treated pouches were injected with both carrageenan and 1 ml of a 1 mg/kg solution of

Figure 1 Immunohistochemical staining of the active form of NF- κ B within rat air pouch tissue. (A–G), NF- κ B staining within rat air pouch tissue at day 1, 2, 3, 6, 14, 21 and 35, respectively, showing the change in distribution of staining cells with time (original magnification $\times 100$).

dexamethasone, these animals were killed three days later. Control animals (non-inflamed pouch) were also killed at day 3. After death the exudate was removed from the pouch and the wall granuloma dissected out. The pouch exudate volume and wet pouch weights were recorded. The excised pouch tissue was fixed in formalin-saline and embedded into paraffin wax blocks.

IMMUNOHISTOCHEMISTRY

Tissue sections (5 μ m thick) were mounted onto slides, dewaxed in xylene and rehydrated through graded alcohols. The tissue sections were then incubated first in a 1% hydrogen peroxide solution in methanol for 10 minutes to quench endogenous peroxidase activity, then in a blocking solution of 1% normal horse serum and 1% bovine serum antigen for 20 minutes to prevent non-specific staining. The primary antibody used was the anti-NF- κ B,

p65 sub-unit at 2.5 μ g/ml (Boehringer Mannheim, UK) and tissue sections were incubated with a 1:200 solution of this antibody overnight at 4°C. This antibody recognises the NLS of the p65 subunit and hence, only activated NF- κ B.⁷ This antibody was detected using a biotinylated secondary antibody (LSAB2 kit, Dako Corporation, UK) and the stain developed using 3,3'-diaminobenzidine (DAB) with nickel enhancement.

Control experiments were performed by (1) omission of the primary antibody and (2) pre-incubation of the primary antibody with the NLS peptide sequence (synthesised by Alta Bioscience, UK).

QUANTIFICATION OF POSITIVELY STAINED CELLS

Cells staining for NF- κ B were counted using a 10 $\times$ 10 grid graticule placed at random in one of two zones in the pouch wall. The intimal zone extended to a depth of about 300 μ m from

Figure 2 Immunohistochemical staining of the active form of NF- κ B. NF- κ B staining within rat air pouch tissue at day 3 in (A) inflamed air pouch tissue stained with phosphate buffered saline (control) (original magnification $\times$ 100), (B) inflamed air pouch tissue stained with pre-absorbed peptide (control) (original magnification $\times$ 100), (C) non-inflamed air pouch tissue (original magnification $\times$ 200), (D) dexamethasone treated inflamed tissue (original magnification $\times$ 200), (E) inflamed air pouch tissue showing the absence of vessel staining (arrows) (original magnification $\times$ 200).

the surface and the sub-intimal zone occupied the area between the intimal zone and the underlying connective tissue (about 1300 μm). A minimum of 50 cells were counted in each area and for each section six areas in each zone were counted. All statistical analyses were performed using an unpaired Student's *t* test.

Results

The model was validated by assessing the volume of exudate and granuloma weight at the time of death. These measurements increase up to day 14 when the inflammatory response is greatest, then reduce with the resolving inflammation (data not shown).

Table 1 shows the mean number of cells staining positive for activated NF- κ B in the intimal and sub-intimal zones of the inflamed air pouch over the 35 day time course and *p* values for comparisons between the two zones. As shown in table 1, there was a greater number of cells staining positive for activated NF- κ B in the intimal zones compared with the sub-intimal zones at earlier time points (see days 1, 2, 3 in table 1). However, after day 3 the pattern was reversed with a greater number of cells staining positive for activated NF- κ B in the sub-intimal zone (see days 6 and 14 in table 1). These differences were significant at all time points ($p < 0.01$) except day 3 ($p = 0.58$). After day 14 the mean number of cells staining positive for activated NF- κ B decreased. This decrease correlated with the resolution of the inflammatory response as seen by reduced granuloma weight and exudate volume. Cell counts for the deeper, sub-intimal zone could not be taken after day 21 as the pouch wall was too thin.

Figure 1 shows examples of photomicrographs of tissue sections stained for activated NF- κ B, from which the cell counts were taken. These illustrate the change in localisation of cells staining positive for activated NF- κ B from the intimal zone (nearest the surface) to the sub-intimal zone (deeper within the tissue) as the time course progresses. At no time point was staining seen in blood vessels (see fig 2E). Control tissue sections stained with either phosphate buffered saline (fig 2A) or pre-absorbed peptide (fig 2B) did not stain for activated NF- κ B.

There were significantly fewer cells staining for activated NF- κ B in both the intimal zone (mean (1SD) 0.021 (0.014) cells per μm^2 ; $p < 0.001$) and sub-intimal zone (0.013 (0.01) cells per μm^2 ; $p < 0.001$) of the non-inflamed pouch when compared with the inflamed pouch at day 3. This can be seen from the photomicrographs by comparing figure 2C (non-inflamed pouch) with figure 2E (inflamed pouch). Similarly, a reduction in the number of cells staining for NF- κ B was seen in both the intimal (0.058 (0.024) cells per μm^2 ; $p < 0.001$) and sub-intimal zone (0.034 (0.018) cells per μm^2 ; $p < 0.001$) of dexamethasone treated pouches when compared with the inflamed pouch at day 3 (also compare fig 2D with fig 2E).

Discussion

This study demonstrates the presence of activated NF- κ B within the inflamed air pouch wall and shows that the activation is associated with the development of the inflammatory response. Activated NF- κ B was identified predominantly in the intimal zone at the earlier time points when the vasculature within the tissue is not well developed. After day 3 this pattern is reversed, possibly because of the rapid development of the vasculature within the subintimal zone and the subsequent infiltration of NF- κ B positive cells.

Previous studies of synovial tissue from patients with rheumatoid arthritis demonstrated staining for activated NF- κ B in both synovial lining cells and synovial microvascular endothelial cells,^{5,6} although staining of the lining seemed to be more predominant in patients with chronic disease.

Recent studies in animal models have shown the induction, during the time course of the inflammatory response, of activated NF- κ B,^{8,9} although only the first of these studies used an immunohistochemical approach. Immunohistochemical staining for NF- κ B in rat adjuvant arthritis synovial tissue revealed staining predominantly in macrophage-like synoviocytes, and around vessels that was attributed to smooth muscle cells.⁸ Clearly, these differences may reflect the nature of the disease processes in the different systems.

The administration of dexamethasone into the pouch at the same time as carrageenan challenge reduced both parameters of inflammation (exudate volume/ granuloma weight, data not shown) and staining for activated NF- κ B in the inflamed pouch. There are various hypotheses on the mechanism of action of glucocorticoid drugs. It has been suggested that glucocorticoids exert their anti-inflammatory activity by inducing the synthesis of I κ B,^{10,11} which blocks the NF- κ B mediated expression of the target pro-inflammatory genes. Another mechanism that may contribute is the direct interaction of the glucocorticoid receptor with NF- κ B.¹² The increase in I κ B synthesis and the interaction with NF- κ B by the glucocorticoid receptor may explain the reduction of NF- κ B activation seen in these pouches.

There is increasing evidence that the activation of NF- κ B is a key step in the inflammatory response within the rheumatoid joint. In addition to corticosteroids, a variety of anti-rheumatic drugs have been shown to inhibit NF- κ B activation in vivo, including aspirin (at high doses)¹³ and tepoxalin.¹⁴ Therefore, activated NF- κ B potentially constitutes an important pharmacodynamic end point in animal model-based studies of novel anti-inflammatory drugs.

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- 1 Baeuerle PA, Henkel T. Function and activation of NF- κ B in the immune system. *Annu Rev Immunol* 1994;12:141–70.
- 2 Selye H. On the mechanism through which hydrocortisone affects the resistance of tissue to injury. *JAMA* 1953;152:1207–13.

- 3 Edwards JC, W Sedgewick AD, Willoughby DA. The formation of a structure with the features of synovial lining by the subcutaneous injection of air: an *in vivo* tissue culture system. *J Pathol* 1981;134:147–56.
- 4 Sedgewick AD, Sin YM, Edwards JCM, Willoughby DA. Increased inflammatory reactivity in newly formed lining tissue. *J Pathol* 1983;141:483–95.
- 5 Marok R, Winyard PG, Coumbe A, Kus ML, Gaffney K, Blades S, *et al.* Activation of the transcription factor nuclear factor kappa B in human inflamed synovial tissue. *Arthritis Rheum* 1996;39:583–91.
- 6 Gilston V, Jones HW, Soo CC-Y, Coumbe A, Blades S, Kaltschmidt C, *et al.* NF-kappa B activation in human knee-joint synovial tissue during the early stages of joint inflammation. *Biochem Soc Trans* 1997;25:518S.
- 7 Kaltschmidt C, Kaltschmidt B, Henkel T, Stockinger H, Baeuerle PA. Selective recognition of the activated form of transcription factor NF- κ B by a monoclonal antibody. *Biol Chem Hoppe Seyler* 1995;376:9–16.
- 8 Tsao PW, Suzuki T, Totosuka R, Murata T, Takagi T, Ohmachi Y, *et al.* The effect of dexamethasone on the expression of activated NF- κ B in adjuvant arthritis. *Clin Immunol Immunopathol* 1997;83:173–8.
- 9 Han ZN, Boyle DL, Manning AM, Firestein GS. AP-1 and NF-kappa B regulation in rheumatoid arthritis and murine collagen-induced arthritis. *Autoimmunity* 1998;28:197–208.
- 10 Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS. Role of transcriptional activation of I κ B- α in mediation of immunosuppression by glucocorticoids. *Science* 1995;270:283–6.
- 11 Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids - inhibition of NF-kappa B activity through induction of I-kappa B synthesis. *Science* 1995;270:286–90.
- 12 Ray A, Prefontaine KE. Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. *Proc Natl Acad Sci USA* 1994;91:752–6.
- 13 Kopp E, Ghosh S. Inhibition of NF- κ B by sodium salicylate and aspirin. *Science* 1994;265:956–9.
- 14 Zhou L, Pope BL, Chourmouzis E, Fung-Leung W-P, Lau CY. Tepoxalin blocks neutrophil migration into cutaneous inflammatory sites by inhibiting Mac-1 and E-selectin expression. *Eur J Immunol* 1996;26:120–9.